Reprint from

Modern Methods of Plant Analysis
New Series Volume 1
Cell Components
Edited by H. F. Linskens and J. F. Jackson

Springer-Verlag
Berlin Heidelberg New York Tokyo

The Marker Concept in Cell Fractionation

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1 Introduction

The intent of this chapter is to provide a practical foundation for anyone interested in performing subcellular fractionation of plant tissue. It is not its purpose to discuss the validity of various markers, since this topic has recently been thoroughly reviewed (Quail 1979). Specific marker assay procedures are given in other chapters of this book and are also available elsewhere (Hall and Moore 1983). Catalogs of "commonly accepted" markers have been published (Bowles et al. 1979; Quail 1979) and should be referred to by the reader.

Historical perspectives and recent reviews have been written on animal (De-Duve 1971, 1975; Morré et al. 1979; Schneider and Hogeboom 1951) and plant cell fractionation (Bowles et al. 1979; Price 1983; Quail 1979) and they should be read before attempting to isolate subcellular components.

2 The Marker Concept

To study biochemical properties and functions of subcellular components, one approach is to disrupt cells and separate various organelles and membranes. The isolated components are identified by their associated markers. A marker is any identification tag which can be used to distinguish one subcellular component from all others after cells are disrupted. It can be a constituent (naturally occurring biochemical entity) of a given component, or it can be exogenously imposed with unique specificity.

2.1 Basic Concepts

An ideal marker is unique to one subcellular component and it is homogeneously dispersed throughout the component population (i.e., the component population is homogeneous in biochemical composition). The ideal marker is likely to be an exception rather than rule (Morré et al. 1979; Quail 1979), since the most commonly used markers appear to have a primary location in one cellular component and a secondary location elsewhere (Morré et al. 1979). For example, NADH cytochrome C reductase activity is associated with both mitochondria and ER, and ATPases have several subcellular locations (Leonard et al. 1973). Other markers

are associated with membranes and also have a soluble counterpart. When markers have more than one location but can be distinguished by selective inhibition or removal of the soluble counterpart, they can still be confidently used to identify particular subcellular components. Since many marker enzymes do not have a single location, predominant localization within a single component is now an accepted criteria for markers (Morré et al. 1979).

Markers may be associated with distinct membrane domains or regions, as has been shown for the Golgi apparatus (Morré et al. 1979, 1983) and, hence, only mark a certain part of an organelle. Regional differences in plasma membranes are also possible (Morré et al. 1979) and it is likely that members of the endomembrane system will exhibit regional domains (Quail 1979). The use of multiple markers will hopefully given a more accurate representation of the distribution of any individual subcellular component.

In general, any marker can be used in one of two modes (Quail 1979). A positive marker is used to show enrichment or purification of a particular component. Negative markers are used to assess the degree of contamination of a particular component by other membranes and organelles.

2.2 Types of Marker

In two recent reviews (Bowles et al. 1979; Quail 1979) markers were classified either as morphological or biochemical. A third category (cytochemical) is added here for clarity. Both morphological and cytochemical markers are monitored and quantitated with the electron microscope.

2.2.1 Morphological

The inherent morphology of some organelles can be used to identify them in subcellular fractions. Except for ER, this technique cannot be used to identify membranous components which vesiculate or fragment during cell disruption. Although morphology provides a specific way to monitor certain organelles, there are several drawbacks when marking in this manner.

The amount of material in thin sections as represented in a micrograph is several orders of magnitude less than that used in biochemical assays (Quail 1979). Quantitation of morphological components (morphometric analysis) is a long and tedious process which requires analysis of a statistically significant number of unbiased electron micrographs. Pelleted fractions are most difficult to analyze because of the stratification observed. Thin sections from various areas of the pellet must be analyzed before the actual contents can be determined. Isolated fractions are best prepared for EM by filtration on Millipore filters (Quail 1979), which eliminates stratification and facilitates their analysis.

The size of the organelles under examination is also a critical factor when evaluating micrographs. For example, if one mitochondrion is consistently seen for every nine chloroplasts in a thin section, one could erroneously conclude that the chloroplast fraction is 90% pure. Taking into account that a mitochondrion can be as small as one tenth size of a chloroplast, the plastid fraction may actually

be 50% pure. With the difficulties aside, morphological markers should be used in conjunction with biochemical markers whenever possible.

2.2.2 Cytochemical

Because the specificity of a cytochemical procedure inherently depends upon the presence of a unique constituent or constituents in a subcellular component, these markers are biochemical in nature. Quantitation of cytochemical markers usually requires examination by electron microscopy and for this reason they are not included in the biochemical category.

Various procedures have been used to selectively stain or selectively enhance staining of ER (Hepler 1981), PM (Quail 1979), and plastid membranes (Hurkman et al. 1979) in intact plant cells. The staining procedure for ER has not yet been applied to isolated fractions, as is the case for PM and plastid membranes (Hurkman et al. 1979, Quail 1979). The question of specificity and the assumptions made when the PM stain is applied to isolated membranes have been discussed (Quail 1979). The same assumptions are made for any cytochemical staining procedure. A minimum control for cytochemical staining procedures requires that intact tissue and isolated fractions be stained at the same time under identical conditions. Cytochemical markers are quantitated by morphometric analysis of electron micrographs and incur the same problems described for morphological markers.

Other cytochemical procedures are not used as markers per se but are used to confirm the subcellular localization of biochemical markers. These procedures involve the subcellular localization of enzymes in intact tissue at the ultrastructural level. Electron-dense reaction products generated directly or indirectly by the enzyme of interest are detected via electron microscopy. The major assumption is that the reaction product remains near or at the site of the enzyme. Cytochemical localization of an enzyme in intact tissue can lend credence to the assignment of its subcellular location by cell fractionation procedures.

2.2.3 Biochemical

Biochemical markers are usually intrinsic constituents of a given organelle or membrane. The most common are enzymatic, and enrichment in marker activity is taken to mean enrichment of the associated membrane or organelle. Nonenzymatic constituents such as chlorophyll (chloroplasts and thylakoids) and cardiolipin (inner mitochondrial membrane) are also used as markers. A third type of biochemical marker is one that is extrinsically imposed on membranes such as surface labeling of the plasma membrane with a radioisotope. Biochemical markers are considerably easier to quantitate than morphological or cytochemical markers. A representative sample of any isolated fraction is easy to obtain and quantitation of any biochemical marker is less subjective than quantitative morphometry.

3 Preservation of Marker Enzyme Activity During Cell Disruption

3.1 Choice of Material

Unless chloroplasts are the object of study, etiolated or nongreen tissues are frequently used for subcellular fractionation since they are low in phenolic compounds as well as chloroplast pigments. Good cell-free systems are characterized by lack of browning and by low absorbance at 260 nm (mainly due to phenolics; Loomis 1974). In addition to phenolics and polyphenol oxidase (PPO) activity, some plant tissues are high in acyl hydrolase activity (Moreau 1985a, b), while others have considerable proteolytic activity (Alpi and Beveers 1981; Caldwell and Haug 1980; Gardner et al. 1971). These hydrolases can contribute to marker enzyme degradation. The extent of these hydrolytic activities in nongreen compared to green tissue is not clear.

The presence of phenolics and PPO activity is readily determined by homogenizing the tissue in basic homogenization medium without additives (see Sect. 3.2). If the crude homogenate turns brown during isolation or after short-term storage, phenolics and PPO activity are likely. There are no quick ways to determine the presence of acyl hydrolase or protease activity. Crude homogenates or crude fractions isolated by differential centrifugation should be assayed for markers immediately after fractionation. The crude fractions should then be stored on ice and marker enzyme activity monitored over a period of time. Loss of marker activity after several hours of storage can indicate a potential acyl hydrolase or protease problem. Several approaches can be used to minimize degradation of membrane constituents and these will be discussed shortly.

3.2 Homogenization Procedure

Historically, plant subcellular fractionation is based on the quantitative approach and techniques employed by animal cell biochemists. Fractionation of plant cells is inherently more difficult because of the presence of a cell wall, the presence of secondary metabolites (especially phenolic compounds), and the presence of hydrolytic activity compartmented in vacuoles and plastids. Conventional approaches to fractionation require fairly harsh homogenization procedures to break open cells, and consequently secondary metabolites and hydrolytic enzyme activity are released when subcellular compartments are ruptured.

Homogenization techniques include razor blade chopping (by hand or mechanically driven chopper), mortar and pestle, ground glass homogenizers (Dounce), Potter-Elvehjem homogenizer, Virtis, Polytron, and Waring blendor. The latter three should not be used on plant tissues that are known to contain high levels of phenolics and PPO activity. Whirling blade mechanisms actually whip air into the homogenate (Loomis 1974) and will provide the oxygen necessary for PPO activity. Mechanically driven razor blade choppers also produce consider-

able frothing and personal experience with potato leaves has shown an enhanced browning of the homogenate when this technique is employed.

The most commonly used cell disruption techniques are razor blade chopping and grinding by mortar and pestle since they produce low to medium shear forces and consequently least damage to intact organelles. These procedures probably produce a low yield of organelles when compared to the total number of organelles present in intact tissue. The report by Jacobsen (1968) indicated that grinding by mortar and pestle produced a low yield (17%) of proplastids in the crude homogenate. The total number of proplastids in the intact etiolated corn leaves was determined two different ways, and all isolated fractions were related back to the total number present before homogenization. The major loss of proplastids was presumably due to unbroken cells which were removed by filtering through Miracloth. Another approach to estimating cell breakage compared the phospholipid content of unground roots to that of the crude homogenate. This report (Fisher and Hodges 1969) indicated that 42% of the membranes were released by mortar and pestle grinding. Even with fairly harsh homogenization procedures, the apparent yield of organelles and membranes from intact tissue appears to be low.

The homogenization procedure may have a direct effect on the distribution of marker enzymes separated by differential centrifugation. With low shear force (razor blade chopping), as much as 60% of PM and ER marker activity (Lord et al. 1972; Nagahashi and Beevers 1978) are pelleted with the crude mitochondrial fraction. With medium shear (mortar and pestle), PM is evenly distributed between the crude mitochondrial and crude microsomal fraction (Leonard and Vanderwoude 1976). Higher shear techniques such as the polytron probably generates smaller microsomal vesicles and consequently, as much as 70 to 80% of the plasma membranes will remain in the post-mitochondrial supernatant (Koehler et al. 1976). The disadvantage of high shear homogenization is that lower yields of intact organelles are probable, which means that the microsomal fraction will have considerable contamination of plastid membranes, mitoplasts, inner and outer mitochondrial membranes, and possibly nuclear membranes.

3.3 Use of Additives in the Homogenization Medium

In many plant tissues, phenolics are mainly responsible for loss of marker enzyme activity. Phenolics can complex with proteins via H-bonds (Loomis and Battaile 1966) and they can also be oxidized by PPO in the presence of oxygen to form quinones (Mayer and Harel 1979). Quinones can readily oxidize functional groups of proteins (Loomis and Battaile 1966) and/or nonenzymatically polymerize (Maillard reaction) to form dark pigments which covalently bond to proteins (Loomis 1974). Phenolics which are released from disrupted cells can be removed by adsorbants (Table 1). The removal of phenolics by insoluble polyvinylpyrollidone is most practical, since bound phenolics can be removed by low speed centrifugation and the remaining supernatant can be fractionated and assayed without concern for interference from the additive. Oxidation of phenolics is prevented by homogenizing in the presence of reducing reagents and/or inhibitors of PPO activity (Table 1).

Table 1. Additives which may be used in the homogenization medium to sustain (protect) marker enzyme activities

Mode of action	Additive or protective agent
Protease inhibition	Phenylmethanesulfonyl fluoride (Alpi and Beevers 1981; Caldwell and Haug 1980; Gardner et al. 1971; Scherer 1981), leupeptin (Alpi and Beevers 1981), glycerol (Alpi and Beevers 1981), iodoacetamide (Alpi and Beveers 1981), p-Cl-mercuribenzoate (Alpi and Beevers 1981)
Lipolytic acyl hydrolase and phospholipase inhibition	Nupercaine (dibucaine) (Bishop and Oertle 1983; Scherer and Morré 1978), BSA (Galliard 1974; Loomis 1974), EDTA (Moore and Proudlove 1983; Philipp et al. 1976), EGTA (Galliard 1974; Miflin 1974), sulfhydryls (Moore and Proudlove 1983), choline and ethanolamine (Scherer and Morré 1978), sodium fluoride (Philipp et al. 1976)
Polyphenolic oxidase inhibition and prevention of browning	Sodium mercaptobenzothiazole (Anderson 1968; Loomis 1974), sodium ascorbate (Anderson 1968; Moore and Proudlove 1983), sodium metabisulfite (Anderson 1968; Loomis 1974; Moore and Proudlove 1983), polyvinylpyrrolidone (Loomis 1974; Loomis and Battaile 1966), BSA (Loomis 1974), thiols and reducing agents (Anderson 1968)
Adsorbs free fatty acids	BSA (Anderson 1968; Galliard 1974; Loomis 1974)
Adsorbs phenolic compounds and tannins	Soluble polyvinylpyrrolidone (Loomis 1974), BSA (Anderson 1968; Loomis 1974), Insoluble polyvinylpyrrolidone (Loomis 1974; Loomis and Battaile 1966)

In addition to phenolics and PPO activity, the hydrolytic activity of lipolytic acyl hydrolases and proteases can present a major problem during membrane isolation. The degradative effect of these enzymes may be somewhat controlled by pH since both types of hydrolases are usually active under acidic conditions (Alpi and Beevers 1981; Galliard 1974). Homogenization should be performed in the cold (0 °–4 °C) with an osmoticum (0.25–0.5 M sucrose, sorbitol, or mannitol) buffered (0.03 to 0.15 M hydrogen ion buffers such as HEPES, TES, MES [1], and Tricine) between pH 7.0 to 8.0. Other additives can be added to the basic homogenization medium to inhibit acyl hydrolase and protease activity (Table 1). Additives should be used with discretion, since it must be determined whether they have a direct effect on marker enzyme activity, have an effect on the assay procedure itself, or interact with one another and consequently interfere with certain enzyme assays (Koundal et al. 1983).

The use of additives can also be counter-productive in another manner. For example, the presence of metabisulfite inhibited PPO activity in tobacco leaves but also produced optimal peptidase activity (Loomis 1974). Our experience with

1 HEPES = N-2-hydroxyethyl piperazine-N′-2-ethane sulfonic acid
MES = (2-[N-morpholino]) ethane sulfonic acid
TES = (N-tris [hydroxymethyl]) methyl-2-aminoethane sulfonic acid
Tricine = (N-tris [hydroxymethyl]) methyl glycine

potato leaves has indicated that sulfhydryls (DTT or β-mercaptoethanol) can retard browning; however, much higher levels of acyl hydrolase activity are maintained (Moreau 1985a).

3.4 Gel Filtration to Remove Soluble Hydrolytic Activity

Regardless of the composition of the homogenization medium, it is necessary to separate membranes and organelles from soluble hydrolases and secondary metabolites as rapidly as possible. Fast separation is usually achieved by differential centrifugation or by centrifugation of unpelleted homogenates directly on sucrose gradients (see Sect. 4.3.2). Recently, several workers (Boller and Kende 1979; Jones 1980; Scherer 1981; Van der Wilden et al. 1980) have used gel filtration as the initial separation step. The crude homogenate is layered on a Sepharose 4B (Boller and Kende 1979; Jones 1980; Van der Wilden et al. 1980) or Sepharose 2B-CL column (Scherer 1981). Apparently, most of the subcellular components pass in the void volume, while soluble hydrolytic enzymes are retarded by the column. This technique has the advantage of removing undesirable soluble enzymes without pelleting organelles; however, very little quantitative data is available showing percent recovery of markers after gel filtration (Van der Wilden et al. 1980) and short separation time may require small columns with small sample size.

Even with rapid separation of membranes from the supernatant, there is no guarantee that soluble enzymes will not adhere to isolated membranes or be trapped inside isolated membrane vesicles. Protoplast preparations may provide a way to isolate various subcellular compartments intact (especially vacuoles and plastids). Protoplasts can be lysed by gentle techniques and the subsequent release of hydrolases and phenolics can be minimized. The use of protoplasts to study subcellular compartments and the problems associated with this approach are covered elsewhere in this book.

4 Methods Used to Separate Markers

4.1 General Approaches to Cell Fractionation

Separation of organelles and membranes is based on differences in size, density, or surface properties. Purification based on differences in surface properties is relatively new and includes such techniques as phase partition (Larsson and Anderson 1979; Yoshida et al. 1983), free flow electrophoresis (Dubacq and Kader 1978; Hannig and Heidrich 1974; Morré et al. 1983), and affinity column chromatography (Schroeder et al. 1982). The application of these techniques to plant tissue needs further evaluation.

The most common approach to subcellular fractionation has been a combination of differential centrifugation (size separation) and density gradient centrifu-

gation. Differential centrifugation is the oldest form of subcellular fractionation and will be discussed first.

4.2 Differential Centrifugation

Low speed centrifugation (250 to 3000 g) is typically used to pellet most of the nuclei (Price 1979), plastids (Jacobson 1968), cell wall fragments (Nagahashi et al. 1985), and unbroken cells. The supernatant is centrifuged at higher forces (6000 to 20,000 g) to sediment most of the mitochondria (Nagahashi and Hiraike 1982) and microbodies which have a similar size. The post-mitochondrial supernatant is centrifuged at high speeds (40,000 to 120,000 g) to pellet microsomes. Microsomal fractions are heterogeneous mixtures consisting largely of ER, PM, Golgi membranes (Koehler et al. 1976; Leonard et al. 1973; Nagahashi and Beevers 1978; Nagahashi and Kane 1982; Philipp et al. 1976), and probably tonoplast. The post-microsomal supernatant contains the "soluble" enzymes [considerable microsomes are still in suspension in 80,000 g supernatants (Koehler et al. 1976) but can be pelleted at 120,000 g (Nagahashi and Hiraike 1982)].

Recently, differential centrifugation has been used primarily to collect large organelles or to obtain a microsomal fraction to use for further purification. In either case, optimum centrifugation conditions should be determined to achieve best separation with minimum cross-contamination. A recent report (Nagahashi and Hiraike 1982) has determined the optimum centrifugal force and centrifugation time for separating crude microsomes from crude mitochondria. Best separation was achieved when lower centrifugal forces (6000 to 8000 *g* for 20 min) were used to pellet mitochondria. These optimum conditions may only apply to tissues homogenized by mortar and pestle and not to tissue homogenized by other techniques.

4.2.1 Preparative vs. Analytical Cell Fractionation

Historically, the goal of preparative fractionation was to isolate and purify a morphologically identifiable component for the subsequent determination of its biochemical and/or physical properties. The major problems with this approach were the lack of criteria used to determine purity (quantitative reliability) and the erroneous assignment of biochemical properties to an incompletely "purified" component (DeDuve 1971).

Analytical cell fractionation focuses on the actual objects of the analyses (biochemical constituent or enzyme activity) and the determination of these entities among all fractions separated from the crude homogenate. Quantitative recovery of markers in all fractions is the characteristic trait of the analytical approach and was insisted upon by Claude (DeDuve 1971) and championed by Schneider and Hogeboom (1951; DeDuve 1971). This approach has been recommended for plant cell fractionation studies (Quail 1979).

4.2.2 Need for Quantitation

To determine the subcellular location of an enzyme, the distribution of its activity and whatever markers being tested should ideally be related back to the total crude homogenate. The reported data should include the total activity and specific activity of all markers, as well as the activity of the enzyme of interest in all isolated fractions. These complete balance sheets are in line with the analytical fractionation approach and are necessary for several reasons. If a substantial increase in total enzyme activity is recovered compared to the original homogenate, the increase could be due to removal of an endogenous inhibitor during subsequent fractionation or the presence of an activator which preferentially associates with a particular fraction. A substantial loss in total activity could be due to the concentration of an endogenous inhibitor in one or more isolated fractions. Recombination of fractions (mixing experiments) can be used to determine which fraction(s) contain the inhibitor or activator, and can provide further insight as to how the activities associated with subcellular compartments are related to the activity of the whole cell.

Assuming complete recovery of the starting activities, the distribution observed can help pare down the possible subcellular sites of the enzyme of interest. If most of the activity is found in the post-microsomal supernatant, the small amount associated with particulate fractions must seriously be considered as an adsorption phenomena (Schneider and Hogeboom 1951).

If most of the enzyme activity is associated with a subcellular component, quantitation of all isolated fractions can be used to help choose a specific crude fraction to further purify (Nagahashi and Beevers 1978). In some cases, a combined crude membrane fraction (Leonard and Vanderwoude 1976; Nagahashi and Baker 1984; Nagahashi and Kane 1982) will be chosen or an unpelleted crude fraction (Lord et al. 1972) will be used for further purification and analysis. In all of these cases, the choice of a crude fraction to purify further was based on initial results from differential centrifugation experiments.

4.2.3 Problems with Complete Quantitation and Interpretation of Data

If the homogenization medium contains additives, they may interfere with biochemical assays performed on original crude homogenates and supernatant fractions. For example, additives such as EDTA in the crude homogenate could interfere with Mg^{2+}-ATPase activity due to the potential chelation of Mg^{2+} in the reaction mix. Other additives such as PVP may give a positive reaction to the Lowry procedure (Pertoft and Laurent 1977). The presence of BSA in the crude homogenate may also lead to falsely elevated protein estimates, since BSA can bind phenolics. In this case, the background protein (BSA content) will not be accurately accounted for and, hence, the apparent specific activities of the crude homogenate or supernatant fractions may be lower as a result of this artifact. The effects of additives on marker enzyme activity as well as assay procedures can be minimized by diluting the crude homogenate in homogenization medium minus the additives. Interference can likewise be minimized in isolated pellets by resuspension in the absence of additives. In some cases, it is possible to dialyze out the

additives before performing enzyme assays. In tissues such as potato leaves or tubers, dialysis cannot be performed fast enough, since marker activity is lost rapidly even at 0 °–4 °C.

In cases where a large percentage of the total crude homogenate activity is soluble, the small percentage associated with subcellular components should not automatically be assumed as artifactual adsorption. A membrane-bound cellulase represented 5–10% of the total cellulase activity isolated from kidney bean abscission zones (Koehler et al. 1976), and 5–8% of the total triose phosphate isomerase activity from various tissues was associated with plastids (Miflin 1974). Enzymes like triose phosphate isomerase that have a cytosolic form as well as a soluble form inside an organelle can confound the distribution pattern of activity in isolated fractions. Enzyme profiles in this case will depend upon the amount of damage done to originally intact organelles.

If the small percentage of total activity associated with particulate fractions is actually due to a soluble contaminant, addition of BSA to the homogenization medium can be effective in preventing the nonspecific adsorption of this activity (Dalling et al. 1972). This treatment is not always effective, and washing the isolated pellets may be necessary to remove soluble contaminants (Elias and Givan 1978). The soluble contaminants removed by washing should be combined with the post-microsomal supernatant to insure accurate bookkeeping.

The pH of the homogenization medium may increase or decrease the adsorption of "soluble" enzymes to crude particulate fractions (Jaynes et al. 1972). At pH 5.0, most of the β-glucosidase activity from sweetclover leaves and bean hypocotyls (Jaynes et al. 1972) was strongly associated with a crude mitochondrial fraction (17,000 *g* for 10 min). At pH 8.5, most of the activity was found in the post-mitochondrial supernatant (Jaynes et al. 1972). Homogenization pH is normally not considered in this light, but one should be aware that acidic or basic conditions may greatly influence the distribution of soluble enzymes during differential centrifugation.

4.3 Linear Density Gradient Centrifugation

Crude fractions separated by differential centrifugation can be further purified in density gradients. Linear gradients are recommended over step or discontinuous gradients because better resolution (as indicated by higher specific activities) can be obtained (Quail 1979).

4.3.1 Density Gradient Material

The most commonly used materials for density gradient centrifugation (Ford et al. 1983) can be grouped under the following categories: (1) sugars and related polymers (sucrose, sorbitol, mannitol, dextran, and ficoll); (2) salt solutions (cesium chloride); (3) colloidal silica suspensions (Ludox, percoll); and (4) iodinated compounds (urografin, renografin, metrizamide, nycodenz). All of these gradient materials have certain properties which make them nonideal. The osmotic effects of sucrose (Price 1982, 1983), anomalous effects of ficoll (Leonard

and Vanderwoude 1976), interaction of metrizamide with proteins and enzymes (Ford et al. 1983), and interference of Percoll with protein analysis (Pertoft and Laurent 1977; Yakmyshyn et al. 1982) and enzyme assays (Yakmyshyn et al. 1982) are examples of these properties.

In spite of its osmotic effects, sucrose is the most widely used gradient material because it is inexpensive, transparent, highly soluble and does not interfere with most marker assays. Although results from sucrose density gradient analysis will be discussed at length, the types of problem encountered, the need for quantitation of markers, and problems in data interpretation will apply to density gradient separation regardless of gradient material used.

4.3.2 Pelleted vs. Unpelleted Overlays

Pelleted fractions isolated by differential centrifugation are further separated by centrifuging in density gradients. Two objections to the use of pelleted overlays have been raised. It has been argued that pelleting is potentially destructive (Quail 1979) and furthermore may enhance nonspecific adsorption of membranes to each other (Ray 1977). The evidence which has led to these objections is unclear. Large intact organelles such as nuclei and chloroplasts may be fragile and break during pelleting and resuspending (Tautvydas 1971), but this is not always the case (Elias and Givan 1978). In contrast, pelleting of microsomes may be nondestructive, since they represent a population of small-sized vesicles derived from broken membraneous structures. Pelleted fractions have the advantage that they can be washed to remove soluble enzymes and additives from the homogenization medium. Resuspended pellets can be applied to density gradients either as an overlay or underlay (flotation method).

If pelleting causes adherence problems, crude particulate fractions can be collected on sucrose cushions to avoid pelleting (Koehler et al. 1976; Ray 1977) or alternatively, unpelleted fractions can be overlaid directly on density gradients (Koehler et al. 1976). This latter method has been used by Lord et al. (1972, 1973) and has been recommended as the method of choice for density gradient centrifugation (Quail 1979). It should be pointed out that the decision by Lord et al. (1972) to use an unfractionated overlay (270 g supernatant) resulted from their initial differential centrifugation experiments. Unfractionated homogenates will provide the greatest amount of soluble enzyme contamination in density gradients and, furthermore, only small volumes can be overlaid on gradients centrifuged in commonly used swinging bucket rotors (SW 25.1, SW 27, SW 28, SW 28.1). The SW 25.2 rotor does have a large capacity and can take up to 30 ml of homogenate over a 30 ml gradient (Fig. 1).

4.3.3 Soluble Enzyme Contamination in Gradients

Soluble enzymes can contaminate sucrose gradients in three different ways. They may nonspecifically adhere to membranes (Dalling et al. 1972; Mandala et al. 1982) and be carried into density gradients. The degree to which the binding is pH-dependent is not known; however, this form of contamination can lead to an erroneous distribution of activity in the fractionated gradient. Plastids are a ma-

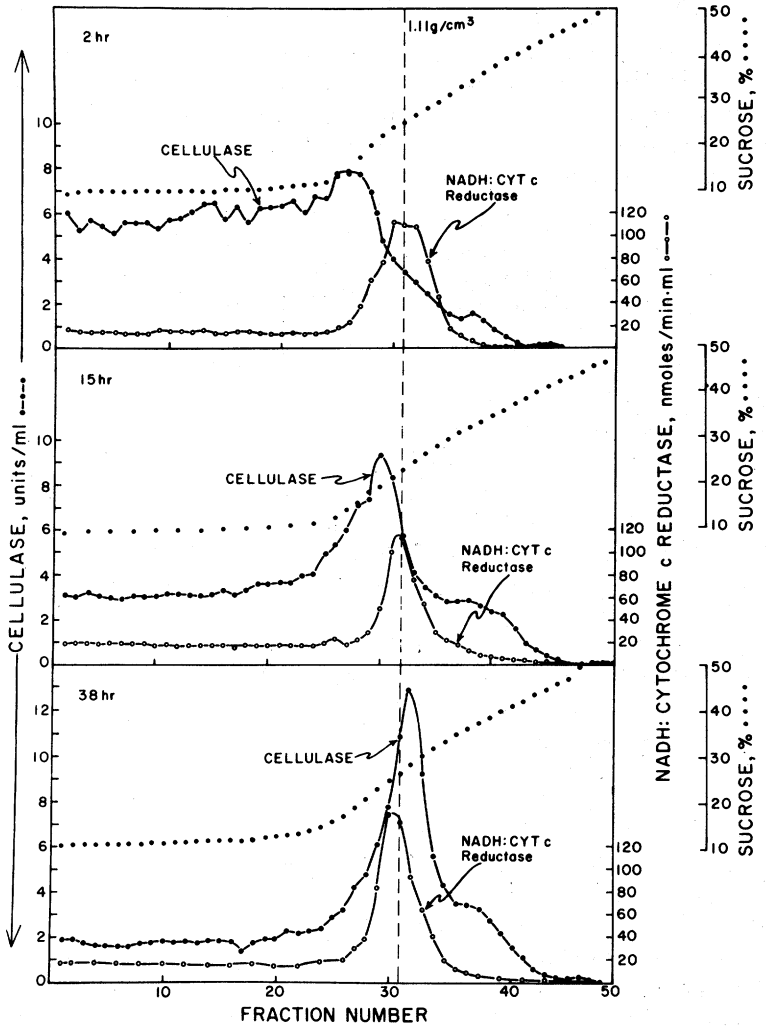

Fig. 1. Sedimentation velocity gradient of peak I (soluble cellulase). An 80,000 g supernatant (30 ml) from the homogenate of bean abscission zones was layered over a linear 15 to 50% (w/w) sucrose density gradient and centrifuged for 2, 15, or 38 h. The three gradients were aligned at a common density of 1.11 g cm^{-3} and plotted as shown. Fractions of 1.2 ml were collected. (Koehler et al. 1976)

jor site of adsorption by soluble enzymes (Dalling et al. 1972; Elias and Givan 1978), and BSA has been used to prevent this binding (Dalling et al. 1972). BSA will not reduce the adsorption of all soluble enzymes and in this situation, repeated washing of crude organelle pellets prior to density gradient centrifugation is necessary to remove soluble enzyme contamination (Elias and Givan 1978).

Secondly, soluble enzymes can enter gradients during prolonged centrifugation (15 h or longer) even when they are not adhering to a membrane or organelle

(Koehler et al. 1976; Lord et al. 1972; Nagahashi and Baker 1984; Nagahashi et al. 1984). This contamination is exacerbated when unfractionated homogenates are used; however, unwashed pellets also carry over considerable soluble enzyme activity. After prolonged centrifugation, soluble enzymes will contaminate light density membranes (Koehler et al. 1976; Lord et al. 1972; Nagahashi and Baker 1984) and will appear to be localized with ER or possibly tonoplast and Golgi membranes.

To determine if enzyme activity near the top of density gradients is actually associated with a light density membrane or is a soluble contaminant, identical linear gradients should be centrifuged for various time periods (Fig. 1) (Nagahashi and Baker 1984; Nagahashi et al. 1985 a, b). If associated with a membrane, the distribution of enzyme activity will not change once the membrane is at equilibrium density. If soluble, the activity will continue to move further into the gradient during extended centrifugation time. Alternatively, differently shaped linear gradients can be centrifuged until membranes are isopycnic. The gradient shape will directly effect the density of a soluble enzyme (Nagahashi et al. 1985 b) but will not effect the equilibrium density of any given membrane.

To minimize this soluble enzyme contamination, Lord et al. (1973) layer a 5 ml step of 20% sucrose on top of a preformed linear sucrose gradient (32–60%). The crude homogenate (5 ml of a 270 g supernatant) is layered on top of the step which retards the movement of soluble enzymes into the gradient during short term centrifugation (4 h). However, "soluble" cytidyl transferase moved further into the gradient with prolonged centrifugation (24 h). This particular gradient design also produces zone-narrowing in the region of the ER, since a normal linear gradient (Lord et al. 1973) shows a much broader peak of ER-associated activity. Unfortunately, for tissues with considerable Golgi bodies and well-developed vacuoles (unlike castor bean endosperm), the gradient-induced zone narrowing (Price 1982) will stockpile tonoplast and Golgi membranes in the region of ER.

Thirdly, soluble enzymes can be entrapped in membrane vesicles during cell disruption. This form of contamination will be greatest in the crude microsomal fraction and will be carried into sucrose gradients to the density of the membrane vesicles which contain the enzyme. Sodium carbonate has been used to remove entrapped soluble enzymes (Fujiki et al. 1982) and this treatment converts closed vesicles to open sheets. Entrapped proteins and peripheral membrane proteins are released in soluble form. It has not been reported whether this process can be reversed to form resealed vesicles from open sheets.

Isolated membranes from density gradients could be treated with sodium carbonate to determine if enzyme activity associated with vesicles is a soluble form inside or a peripheral membrane protein. If the activity remains with the membranes after treatment, it is likely to be an integral protein.

4.3.4 Equilibrium Density Centrifugation (Isopycnic Conditions)

Maximum separation of subcellular components in density gradients can only be achieved when membranes are at their equilibrium density. Although little attention has been given to this fact, a few reports (Koehler et al. 1976; Leonard and

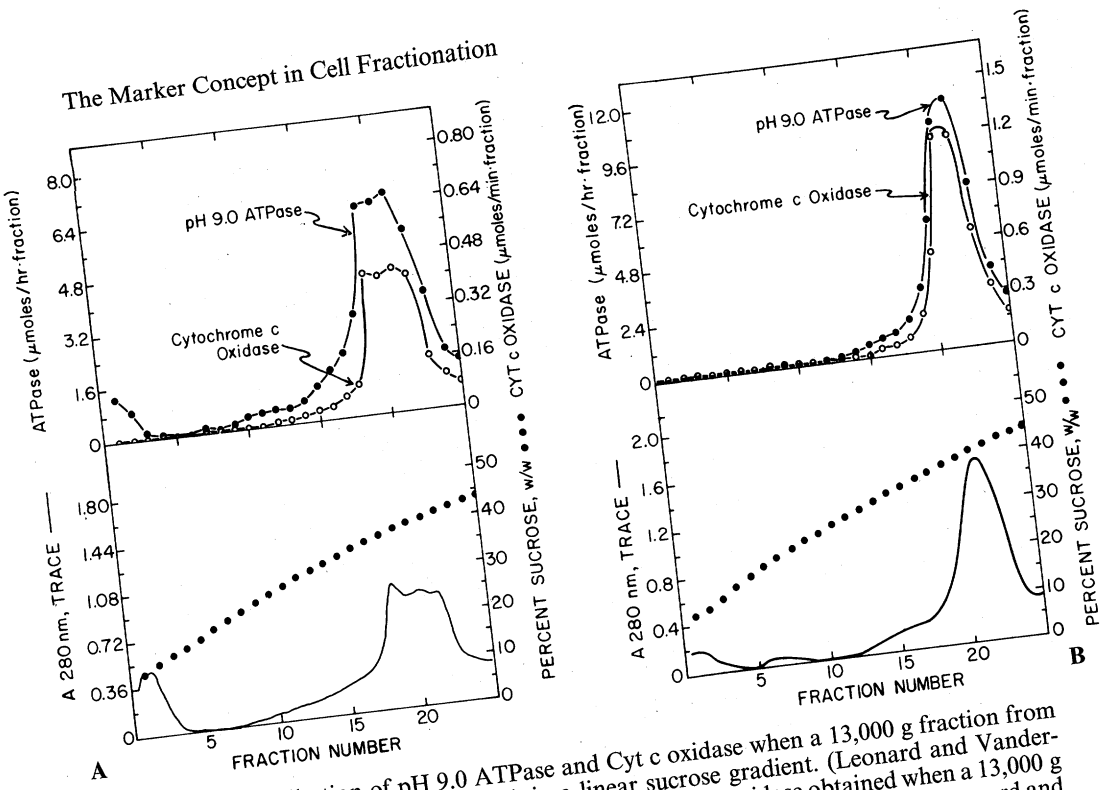

Fig. 2. A Distribution of pH 9.0 ATPase and Cyt c oxidase when a 13,000 g fraction from corn roots was centrifuged for 1.5 h in a linear sucrose gradient. (Leonard and Vanderwoude 1976) **B** Distribution of pH 9.0 ATPase and Cyt c oxidase obtained when a 13,000 g fraction from corn roots was centrifuged for 15 h in a linear sucrose gradient. (Leonard and Vanderwoude 1976)

Vanderwoude 1976; Nagahashi and Baker 1984) have indiciated that membranes and organelles do not reach isopycnic conditions (as judged by marker enzyme distribution) after centrifugation for 1.5 to 2 h at high speeds (80,000 to 85,000 *g*). For the mitochondrial (cyt c oxidase, pH 9.0 ATPase), plasma membranes (pH 6.5 K^+-ATPase), and ER (antimycin A insensitive NADH cyt c reductase) markers (Nagahashi and Baker 1984), short term centrifugation (Figs. 1, 2 A, 3 A) resulted in broad peaks of activity with corresponding broad peaks of absorbance at 280 nm (Figs. 2A, 3 A). These data are interpreted to indicate that not all members of a given component population have reached isopycnic conditions. Afer 15 h of centrifugation, the markers are much sharper in resolution (Figs. 1, 2 B, 3 B). Further centrifugation up to 38 h (Fig. 1) or 40 h (Nagahashi and Baker 1984) did not change the resolution of the ER marker, regardless of whether pelleted overlays or unpelleted overlays were used. Consistent with these results is a recent report on the subcellular fractionation of guinea pig enterocytes (Ford et al. 1983). Mitochondria, lysosomes, and plasma membranes all showed much tighter banding patterns in sucrose gradients centrifuged for 16 h (100,000 *g*) compared to 2 h (30,000 *g*). This report (Ford et al. 1983) also showed differences in membrane densities when short- and long-term centrifugation were performed with metrizamide and nycodenz gradients.

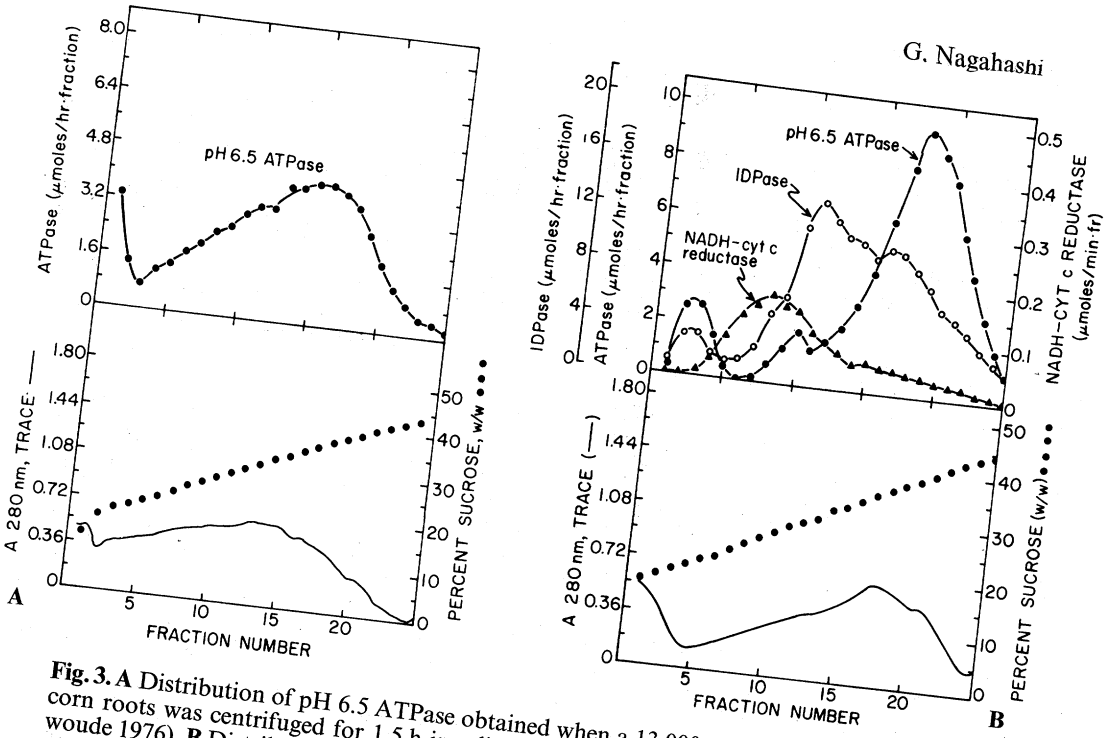

Fig. 3. A Distribution of pH 6.5 ATPase obtained when a 13,000 to 80,000 g fraction from corn roots was centrifuged for 1.5 h in a linear sucrose gradient. (Leonard and Vanderwoude 1976). **B** Distribution of pH 6.5 ATPase, latent IDPase, and NADH cyt c reductase obtained when a 13,000 to 80,000 g fraction from corn roots was centrifuged for 15 h in a linear sucrose gradient. (Leonard and Vanderwoude 1976)

It is not known whether centrifugation for 6 to 8 h will be the minimum time necessary to reach equilibrium conditions. With the common type of swinging bucket rotors, centrifugation for 6 to 8 h presents an inconvenient time period since homogenization, centrifugation, fractionation, and enzyme assays cannot be performed during the same work day. Preparation and analysis of gradients centrifuged for 2 h or less can be done in 1 day and, hence short-term centrifugation is used by many workers. Overnight centrifugation (15 h) is recommended, since maximum separation of markers is insured and a complete day for marker assays is available.

3.5 Other Factors Which Influence Marker Enzyme Profiles ross a Gradient

level of membrane protein will affect the detection of enzyme activity espe- if the assay procedure is rather insensitive. At low protein levels in sucrose nts, some peaks of enzyme activity are not readily detectable, but become ble when the protein level in the gradient is increased (Nagahashi et al. n these cases, marker assays may need to be modified (larger aliquots as- longer incubation periods) before activity can be detected.

Another factor which can influence marker enzyme activity is the presence of additives in the gradient. EDTA and Mg^{2+} can alter marker enzyme and protein profiles when added to sucrose gradients. When ribosomes are retained ($+Mg^{2+}$), RER markers have a density range of 1.15–1.18 g/cm^{-3} in sucrose gradients (Quail 1979). The density of ER markers when ribosomes are removed (+EDTA) is much lighter (1.10–1.12 g/cm^{-3}). Other additives such as buffers and sulfhydryls may help stabilize marker activity especially if prolonged centrifugation is used. Higher levels of marker activity may be sustained with protective reagents and may result in more accurate recoveries in gradient fractions.

Finally, the resolution of markers in density gradients can be diminished by the collection of large fractions, as well as by inappropriate centrifugation conditions (Sect. 4.3.4). Best resolution has been achieved when small fractions (0.6 ml to 1.5 ml) are collected during fractionation of large gradients (38 to 60 ml) (Koehler et al. 1976; Leonard and Vanderwoude 1976; Lord et al. 1972; Nagahashi and Baker 1984; Nagahashi and Kane 1982).

4.3.6 Need for Quantitation and Lack of Negative Marker Activity

The total activity and specific activity of markers before and after density gradient centrifugation should be determined. Quantitation of all gradient fractions including the material which pellets through the gradient should be determined (Quail 1979) and related back to the crude overlay. Incomplete recovery of marker activity after density gradient fractionation can be due to any of the reasons discussed earlier (Sect. 3.3 and 4.2.2).

Markers used in the negative mode should be monitored to determine the degree of contamination of a "purified" fraction. Lack of negative marker activity is usually interpreted to mean no contamination by membranes carrying the negative marker. This is only one possibility, since any of the following is also possible: (1) the negative marker may be unstable and activity is lost during gradient centrifugation; (2) the negative marker chosen may not be ubiquitous; (3) low protein levels in the gradient may prevent detection of the negative marker; (4) the enzyme assay is performed incorrectly. In these cases, membranes or organelles contaminating the "purified" fraction are actually present but cannot be detected. The use of multiple markers combined with morphological examination of the isolated fraction will provide a clearer picture of the degree of contamination in "purified" fractions.

5 Concluding Remarks

The scope of this article has been to present basic concepts about markers and suggest a basic approach to plant cell fractionation. The value of differential centrifugation experiments cannot be overemphasized. Membrane fractions separated by simple centrifugation experiments can be used to work out marker assay procedures, determine the stability of marker enzymes, and determine if PPO and

hydrolytic activity are present. Even with relatively poor resolving power, analytical fractionation by differential centrifugation can provide quantitative information about the distribution of biochemical properties over a number of fractions which together represent the whole tissue.

To study biochemical functions through cell fractionation is no easy task, and should be performed with as much rigor as technically possible. The major problems which occur during cell fractionation and the potential erroneous conclusions drawn from experiments without appropriate controls have been discussed. The approach proposed and the problems of data analysis discussed should be taken neither as gospel nor the opinions of others, but only as the views of this author.

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